Biochimica et Biophysica Acta, 976 (1989) 94–101
Elsevier

BBABIO 43041

Reactions related with $\Delta\tilde{\mu}_{H^+}$-dependent activation of the chloroplast H^+-ATPase

Detlev Lohse and Heinrich Strotmann

Institut für Biochemie der Pflanzen, Heinrich Heine Universität Düsseldorf, Düsseldorf (F.R.G.)

(Received 20 February 1989)

Key words: Chloroplast; ATPase, H^+-; Enzyme activation; Photophosphorylation; Nucleotide binding

The process of $\Delta\tilde{\mu}_{H^+}$-dependent activation of the chloroplast H^+-ATPase was studied by following initial kinetics of light-induced release of tightly bound labeled nucleotides from CF_1 of pre-loaded thylakoids. The following results were obtained. (1) The initial rate of light-induced nucleotide release is increased by medium ADP and further accelerated by the simultaneous presence of medium phosphate. In the latter case a biphasic time-course is observed, indicating a fast activation of up to 30% of the ATPase followed by a slow activation of the rest. The rapid initial phase is absent when the medium contains ADP only. Phosphate alone does not stimulate light-induced nucleotide release. (2) During the first 20–30 ms of illumination in the presence of ADP and P_i the rates of nucleotide release and photophosphorylation are identical, suggesting a close relationship between the two processes. The initial rates of both reactions are strongly inhibited by valinomycin (in presence of K^+), indicating the significance of a membrane potential as the driving force. In particular the initial ADP + P_i-stimulated nucleotide release is inhibited by DCCD. The tightly bound ADP found in deenergized thylakoids is not the initial phosphoryl acceptor in photophosphorylation. (3) Omission or replacement of Mg^{2+} by Ca^{2+} and substitution of phosphate by phosphate analogues permits the conclusion that maximal stimulation of light-induced nucleotide release requires binding of P_i and ADP-Mg rather than ATP formation at other sites of the enzyme. (4) The effect of varying medium ADP and P_i concentrations on the initial velocity of light-induced nucleotide release reveals two different substrate constants for each of the two compounds. The results suggest participation of two functionally linked free centers in acceleration of nucleotide release. Successive occupation of these two centers with ADP and P_i executes a negative cooperative effect on the third, nucleotide-containing site. The above-mentioned results are explained on the basis of a model employing three principally identical catalytic centers which are occluded in the inactive state of the enzyme. One of them is occupied with ADP or ATP, two are non-occupied. Opening of the three centers by $\Delta\tilde{\mu}_{H^+}$-linked conformational changes renders nucleotide dissociation and interaction of medium substrates with the free sites. Binding of ADP and P_i and simultaneous forced dissociation of the former tightly bound nucleotide are regarded as predisposing steps to establish the functional order of the sites involved in catalytic turnover.

Introduction

Photophosphorylation is energetically driven by a transmembrane electrochemical proton gradient. Coupling between proton flux and ATP formation requires operation of a proton-translocating ATPase, CF_0CF_1, which is a constituent of the thylakoid membrane. The subunit composition of the catalytic CF_1 moiety is $\alpha_3\beta_3\gamma\delta\epsilon$ [1–3]. As β (presumably together with α) contains binding sites for adenine nucleotides [4–7], three catalytic centers have to be assumed to be present per ATPase molecule. Enzymatic models proposing a concerted function of the three centers in catalysis of reversible ATP formation have been established by Boyer and his colleagues ("three site energy linked binding change mechanism") [8] and by Mitchell ("rolling well and turnstile mechanism") [9]. A common feature of both hypotheses is the sequential alteration of the three catalytic centers during enzymatic turnover. In the energy-linked binding change mechanism conformational alterations leading to changes of substrate and

Abbreviations: AdN, adenine nucleotide; Chl, chlorophyll; DCCD, *N,N'*-dicyclohexylcarbodiimide; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; P_i, inorganic orthophosphate; PMS, phenazine methosulfate; TLC, thin-layer chromatography; TNP-ADP, 2'(3')-*O*-(2,4,6-trinitrophenyl)-adenosine 5'-diphosphate; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine.

Correspondence: H. Strotmann, Institut für Biochemie der Pflanzen, Heinrich Heine Universität Düsseldorf, Universitätsstraße 1, D-4000 Düsseldorf, F.R.G.

product affinities are regarded as the energy-dependent steps, whereas the chemical reaction of phosphoanhydride bond formation at the catalytic site is thought to proceed spontaneously with an equilibrium constant close to 1. It was shown by ^{18}O exchange that release of the newly formed ATP from one catalytic site is facilitated by binding of the substrates ADP and P_i to a second catalytic site [10], a result which suppports the cooperation of at least two catalytic centers.

The chloroplast H^+-ATPase is strictly regulated. The enzyme is catalytically inactive in dark-adapted chloroplasts [1–3], but is transferred into an active state upon thylakoid energization by light or artificial gradients [11–13]. The process of enzyme activation is related with liberation of one adenine nucleotide per ATPase which is tightly bound to CF_1 in the inactive ground state [12–14]. On the other hand, deactivation of the pre-activated ATPase is greatly enhanced by tight binding of ADP [14,15]. After deenergization of thylakoids which were allowed to phosphorylate in the light, part of the tightly bound nucleotides may be ATP [16–18]. Recently tightly bound ADP was demonstrated to be located at a site which was previously identified as a catalytic site [19]. These results lead to the interesting conclusion that the same type of sites can switch between catalytic and regulatory function. Although up to three more non-catalytic nucleotide binding sites were detected in isolated CF_1 [7], their functional meaning remains obscure.

Light-induced release of tightly bound nucleotides is accelerated by the presence of ADP or ATP in the medium [20,21], indicating cooperative interactions of the medium nucleotides with other free sites. In the present paper initial kinetics of light-induced mobilization of tightly bound nucleotides were investigated in the absence or presence of medium nucleotides and phosphate to obtain detailed insight into the cooperation of the involved sites in the pre-steady-state phase of photophosphorylation. They give an imagination of the steps involved in the transition of a resting to a working ATPase.

Methods

Isolated thylakoids were prepared from spinach leaves as in Ref. 20. Loading of the membrane-bound ATPase with [^{14}C]ADP was carried out as described [22] with 10 μM [^{14}C]ADP at a chlorophyll concentration of about 0.5 mg/ml. The reaction mix was illuminated for 1 min at 20 °C with 300 $W \cdot m^{-2}$ and then kept in the dark for 5 min before centrifugation and washing of the membranes [22].

Light-induced release of the tightly bound ^{14}C-labeled nucleotides from loaded thylakoids was performed in small cylindrical glass vessels with magnetic stirring device which could be illuminated from the top. Temperature was kept at 20 °C by insertion of the reaction vessels in a temperature-controlled metal bloc. Illumination time was adjusted by an electronic shutter placed in the light beam. Intensity of the white light usually was 600 $W \cdot m^{-2}$. The medium contained 25 mM Tricine buffer (pH 8.0), 50 mM NaCl, 5 mM $MgCl_2$, 50 μM PMS, loaded thylakoids corresponding to 50–100 μg chlorophyll/ml and the components indicated in the legends. Changes of the medium composition are also indicated in the legends. The total volume was 0.5 ml. After brief temperature equilibration in the dark, a light pulse of defined length was given. Immediately after the end of the flash the reaction was quenched with 0.2 ml of a quench solution containing unlabeled ADP and FCCP [23] to yield final concentrations of 1 mM and 50 μM, respectively. The quenched reaction mix was centrifuged and aliquots of the supernatants were analyzed for radioactivity [20]. Chromatographic separation of nucleotides of supernatants or $HClO_4$ extracts of thylakoids were carried out by TLC [24].

Photophosphorylation was measured under the same conditions with the same set-up but with thylakoids preloaded with unlabeled ADP. The inorganic phosphate was labeled with ^{32}P. Following the light flashes, the thylakoids were rapidly deproteinized by $HClO_4$ (final concentration, 0.5 mM). Organic ^{32}P-labeled phosphate was separated by precipitation of the excess inorganic labeled phosphate [25].

Results

Nucleotide loading of the membrane-bound ATPase by illumination of thylakoids with [^{14}C]ADP results in the incorporation of one nucleotide molecule per CF_1 molecule [20]. About 80% of the 'tightly bound'-labeled nucleotide is recovered as ADP; however, 20% may be ATP even when the loading procedure is carried out in the absence of added phosphate. The bound ATP may result from phosphorylation with endogenous P_i. The phosphate content of washed thylakoids was determined as 80 nmol/mg chlorophyll; most of it seems to be located in the thylakoid lumen and gets available for phosphorylation upon illumination [26]. The content of tightly bound labeled ATP may be increased when loading with [^{14}C]ADP in the light is carried out in the presence of 5 mM phosphate [27]. Since formation of tightly bound ATP is not abolished by the presence of a hexokinase system [28], the site of its synthesis is likely to be the same as the site where the tightly bound ATP is located. The percentage of tightly bound ATP is reduced to almost zero, when thylakoids are supplied with [^{14}C]ADP after preillumination and decay of the proton gradient (Table I). Under those conditions the total amount of tightly bound labeled nucleotides is lower.

TABLE I

Patterns of tightly bound labeled adenine nucleotides after loading of thylakoids with ^{14}C-labeled ADP or ATP

Experiment 1

1 min illumination with either 5 μM [^{14}C]ADP or 5 μM [^{14}C]ATP. After 5 min in the dark, the membranes were centrifuged and washed as described in Methods. The nucleotides of the $HClO_4$ extracts were separated by TCL.

Incubation with:	nmol [^{14}C]AdN tightly bound/mg Chl			
	AMP	ADP	ATP	total
[^{14}C]ADP	0.04	0.81	0.20	1.05
[^{14}C]ATP	0.03	0.80	0.24	1.07

Experiment 2

9.4 μM [^{14}C]ADP was present either during 1 min illumination or added 1 min after preillumination. The thylakoids were subsequently kept in dark for 5 min.

Incubation with [^{14}C]ADP:	nmol [^{14}C]AdN tightly bound/mg Chl			
	AMP	ADP	ATP	total
In the light	0.015	0.676	0.095	0.786
After preillumination	0.020	0.403	0.005	0.482

In the following, time-courses of light-induced release of the previously incorporated labeled nucleotides are studied in media of different composition. The results are largely independent of the pattern of the nucleotides bound; hence it seems that tightly bound ADP and tightly bound ATP behave quite similarly. Fig. 1 shows the kinetics of nucleotide release in light flashes from 10 ms to 1 s. In three different experiments the reaction was either carried out in the absence and presence of 5 mM P_i (a), in the absence and presence of 20 μM ADP (b), or in the presence of 20 μM ADP ± 5 mM P_i (c). In accordance with earlier reports on release kinetics at lower time resolution [20], ADP alone is found to increase the initial rate of adenine nucleotide

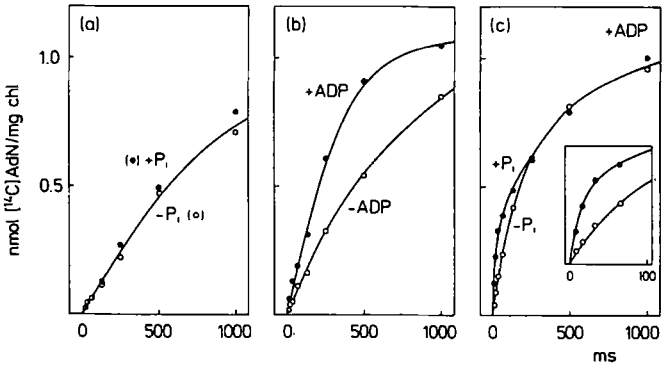

Fig. 1. Kinetics of light-induced release of tightly bound ^{14}C-labeled adenine nucleotides in medium ± 5 mM phosphate (a), ± 20 μM ADP (b) and 20 μM ADP ± 5 mM phosphate (c). Thylakoids were loaded with [^{14}C]ADP as described in Methods. The contents of labeled nucleotides were 0.985 (a), 1.312 (b) and 1.204 nmol/mg chlorophyll (c).

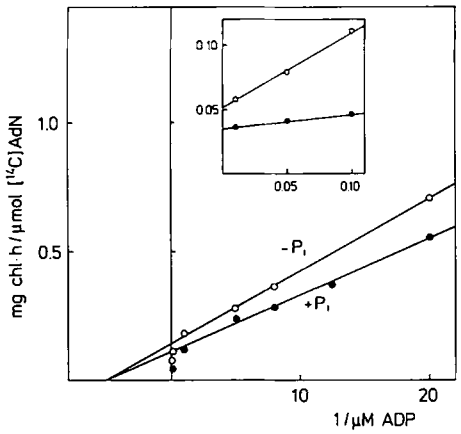

Fig. 2. Rates of light-induced release of tightly bound labeled nucleotides as function of medium ADP concentration in the absence and presence of 5 mM phosphate (double-reciprocal plot). The higher ADP concentration range is resolved in the inset. The rates were calculated from initial kinetics (0–33 ms). The loaded membranes contained 0.94 nmol [^{14}C]AdN/mg chlorophyll.

liberation in the light, whereas P_i alone has no stimulating effect. With or without medium ADP monophasic hyperbolic time-courses are observed. In contrast, biphasic release kinetics are observed in the presence of ADP + P_i. A rapid initial release phase which is terminated after about 50 ms, is followed by slow nucleotide release with a half-time of about 300 ms. The time-course of the slow phase is similar to release kinetics observed in the presence of ADP alone. The amplitude of the rapid initial reaction is 1/4 to 1/3 of the total amount of bound nucleotides. Under phosphorylating conditions biphasic nucleotide release was previously reported by Gräber et al. [12]; our results show that the presence of both, medium ADP and P_i is a prerequisite. Experiments with ATP and a recycling pyruvate kinase system in the medium indicated absence of the rapid phase of nucleotide release irrespective of the simultaneous presence of P_i (results not shown).

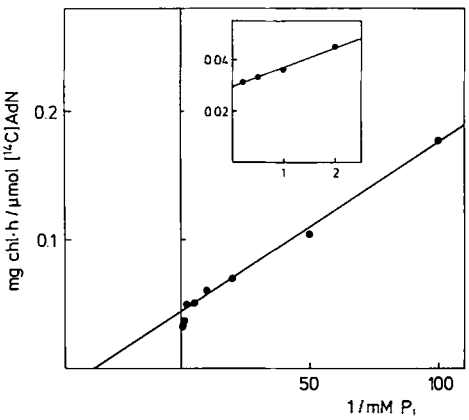

Fig. 3. Rate of light-induced release of tightly bound labeled nucleotides as function of the medium phosphate concentration at 100 μM medium ADP (double-reciprocal plot). The thylakoids contained 1.160 nmol [^{14}C]AdN/mg chlorophyll. Further details as in Fig. 2.

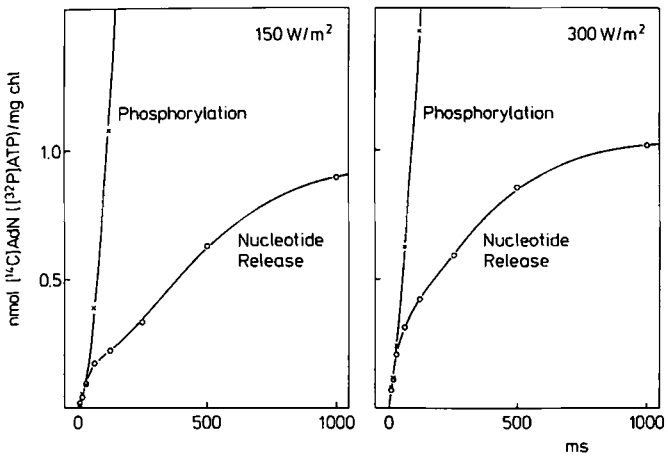

Fig. 4. Initial kinetics of light-induced release of labeled adenine nucleotides and of photophosphorylation at 150 and 300 W/m² measured in parallel experiments under identical conditions. The medium contained 20 μM ADP and 100 μM phosphate. The labeled thylakoids contained 1.25 nmol [^{14}C]AdN/mg chlorophyll.

Initial rates of nucleotide release were measured as function of the medium ADP concentration in the absence and presence of phosphate. Double-reciprocal plots (Fig. 2) reveal that each of the two concentration curves can be subdivided into two hyperbolic reactions, one with a high and one with a low affinity for medium ADP. Estimation of the substrate constants for the high-affinity processes yields identical values in the absence and presence of P_i ($K_{(ADP)1} = 0.2$ μM), whereas the constants of the low-affinity reaction differ by a factor of 3. In the absence of P_i $K_{(ADP)2}$ is 10 μM but in the presence of P_i $K'_{(ADP)2}$ is 3 μM. Similarly the rate of release was investigated as function of P_i concentration at excess ADP (Fig. 3). Again two different reactions can be discriminated by the double-reciprocal plot. The respective substrate constants are $K_{(P_i)1} = 30$ μM and $K_{(P_i)2} = 250$ μM.

In Fig. 4 kinetics of nucleotide release in the presence of ADP and P_i are compared with kinetics of ATP formation (including free plus bound ATP) obtained in parallel measurements under identical experimental conditions. The thylakoids were illuminated with two different light intensities. In the first 20–30 ms the rates of nucleotide release and phosphorylation are identical. The amplitude of the initial rapid release phase increases with light intensity. While the rate of nucleotide release decreases after 50 ms, photophosphorylation is further accelerated and attains a steady rate after about 100 ms in these experiments. The increase of the rate of phosphorylation with time of illumination, which has not been observed in experiments with methylviologen as electron acceptor [29], may be referred to partial membrane damage due to the pretreatment of the thylakoids which requires repeated washes following nucleotide loading. Table II indicates that the initial rates of nucleotide release and photophosphorylation as functions of ADP concentration are identical, too.

TABLE II

Initial rates of light-induced release of tightly bound nucleotides and photophosphorylation as functions of medium ADP concentration

The medium contained the indicated concentrations of ADP and 1 mM phosphate. Initial rates were calculated from kinetics between 0 and 33 ms. The content of labeled nucleotides of the thylakoids used in release measurements was 0.893 nmol/mg chlorophyll.

Medium ADP (μM)	Nucleotide release (μmol [^{14}C]AdN/ mg Chl per h)	Phosphorylation (μmol [^{32}P]ATP/ mg Chl per h)
0	0.8	0.8
2	5.6	5.9
5	19.1	18.4
10	25.2	25.2
50	39.6	36.4

Fig. 5a shows that valinomycin in the presence of K^+ suppresses the rapid phase of nucleotide release; this effect of valinomycin is matched by a corresponding initial inhibition of phosphorylation [29]. The result demonstrates the significance of a membrane potential as the driving force for both reactions during the initial period of energization. The inhibitory effect of DCCD (Fig. 5b) may be taken as evidence that H^+ translocation through CF_0 is necessary for ATPase activation and related nucleotide release. Parallel measurements of the DCCD effect on initial nucleotide release and phosphorylation (not shown) indicated the same dependence on DCCD incubation time.

The preceding results demonstrate the close functional relationship between initial phosphorylation and initial nucleotide release in the presence of ADP and P_i in the medium. Since the tightly bound nucleotides are located on modified catalytic sites [19] and the majority

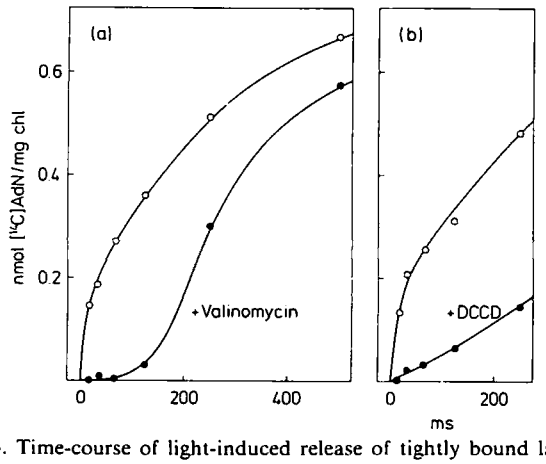

Fig. 5. Time-course of light-induced release of tightly bound labeled nucleotides in the absence and presence of 1.2 μM valinomycin (a) or 20 μM DCCD (b). The medium contained 500 μM phosphate and 10 μM ADP (a) or 500 μM phosphate and 50 μM ADP (b); in (a) NaCl was replaced by 50 mM KCl. The inhibitors were added as ethanolic solutions (2% final ethanol concentration). The DCCD preincubation time was 30 min. The contents of ^{14}C-labeled nucleotides were 0.82 (a) and 1.005 nmol/mg chlorophyll (b), respectively.

TABLE III

$^3H/^{32}P$ double labeling of ATP formed during the initial light phase

The medium contained 6.6 μM [^{3}H]ADP and 300 μM [^{32}P]P_i. The specific radioactivities were $4.44 \cdot 10^{15}$ dpm/mol (^{3}H) and $2.24 \cdot 10^{15}$ dpm/mol (^{32}P). Chlorophyll concentration during the reaction was 0.124 mg/ml. After light flashes of the indicated lengths, thylakoids were deproteinized by $HClO_4$. In two parallel aliquots ATP was separated by TLC (Chrom. I and II). Thylakoids were preloaded with unlabeled ADP (10 μM) which was added after 1 min preillumination.

Illumination time (ms)	molar ratio $^{32}P/^3H$ in ATP	
	Chrom. I	Chrom. II
8	0.95	0.93
33	0.93	0.87
67	1.00	0.93
125	1.28	0.88
250	0.98	0.86
500	1.35	1.08
1000	1.04	1.08

of them consist of ADP, this ADP could be the initial phosphoryl acceptor in photophosphorylation. Actually the energy-independent formation of tightly bound ATP from tightly bound ADP was shown to occur on thylakoids as well as isolated CF_1 at high phosphate concentration and low pH [43,44]. Our results, however, give no indication that the tightly bound ADP under the employed conditions is phosphorylated during the pre-steady-state phase of photophosphorylation. Analysis of the ^{14}C-labeled products released into the medium during the first 50 ms of illumination showed essentially the same pattern as the nucleotides present on the membranes before illumination. In experiments employing thylakoids preloaded with unlabeled ADP in a medium containing [^{3}H]ADP and [^{32}P]P_i, the newly formed ATP was found to contain both isotopes at an 1 : 1 ratio from the beginning of illumination throughout the course of the experiment (Table III). If the unlabeled tightly bound ADP were the initial phosphate acceptor, the ratio $^{32}P/^3H$ in the ATP should decrease with illumination time. These findings are in agreement with results reported in the literature [30]. They permit the firm conclusion that the tightly bound nucleotide leaves the enzyme unchanged when the thylakoids are energized by light flashes; the simultaneously formed ATP originates from medium ADP and medium phosphate.

The obvious kinetic correlation between nucleotide release and phosphorylation in the initial light phase raises the question whether binding of the medium substrates ADP and P_i is sufficient or ATP formation on other sites of the enzyme is necessary to facilitate release of the tightly bound nucleotide. To decide between these alternatives, Mg^{2+} as an essential cofactor of photophosphorylation was omitted or replaced by

TABLE IV

Initial rates of light-induced nucleotide release as affected by Mg^{2+} and Ca^{2+}

The concentrations of the indicated medium compounds were 98 μM ADP, 0.5 mM P_i and 5 mM $MgCl_2$ or $CaCl_2$. The [^{14}C]AdN content of the thylakoids was 0.922 nmol/mg chlorophyll. The initial rates were calculated from kinetic measurements (0–1000 ms).

Compounds present in the medium:	Initial rate of nucleotide release (μmol [^{14}C]AdN/mg Chl per h)
ADP–P_i–Mg^{2+}	18.0
ADP–P_i–Ca^{2+}	9.2
ADP–P_i	5.9
P_i–Mg^{2+}	1.4
P_i–Ca^{2+}	1.8
P_i	1.4

Ca^{2+} which does not support phosphorylation. For full acceleration of nucleotide release indeed Mg^{2+} in addition to medium ADP and P_i is necessary. However, about 50% stimulation is obtained by employing Ca^{2+} instead of Mg^{2+} (Table IV). Since Ca^{2+} as well as Mg^{2+} can form a metal ion complex with ADP, we may conclude that CaADP although being inactive as phosphoryl acceptor may nevertheless be adopted in an active site of the ATPase [31] and thereby effect acceleration of release of the nucleotide from the former tight binding site. Competitive inhibition of photophosphorylation by arsenate, sulfate and sulfite suggests that the phosphate binding site of the chloroplast ATPase interacts with those compounds [32,33]. They were tested as phosphate analogues in acceleration of light-induced nucleotide release. Table V shows that arsenate is nearly as effective as phosphate while sulfate and sulfite exhibit about 50% efficiency; thiosulfate does not replace phosphate. The results suggest that formation of ATP at another catalytic site is not essential for acceleration of nucleotide release. This conclusion is supported by the previous finding that the ADP analogue TNP-ADP,

TABLE V

Initial rates of light-induced nucleotide release in the presence of ADP and phosphate or phosphate analogues

The concentration of ADP was 10 μM, phosphate or phosphate analogues were present at 5 mM. The [^{14}C]AdN content of the thylakoids was 1.27 nmol/mg chlorophyll. Rates were calculated from kinetic measurements (0–1000 ms).

Anions present in the medium:	Initial rate of nucleotide release (μmol [^{14}C]AdN/mg Chl per h)
–	15.5
Phosphate	34.5
Arsenate	33.5
Sulfate	26.9
Sulfite	24.8
Thiosulfate	7.9

TABLE VI

Effect of phlorizin on the initial rates of light-induced nucleotide release and photophosphorylation

The reaction medium contained 100 μM ADP, 1 mM P_i and the indicated concentrations of phlorizin. The [^{14}C]AdN content of the preloaded thylakoids was 1.016 nmol/mg chlorophyll. Initial rates were gained from kinetics between 0 and 33 ms.

Phlorizin concentration (mM)	Nucleotide release (μmol [^{14}C]AdN/ mg Chl per h)	Phosphorylation (μmol [^{32}P]ATP/ mg Chl per h)
0	27.0	33.0
0.1	23.2	26.4
0.5	20.3	–
1	7.6	12.2
5	3.5	1.7
10	0.5	0.0

which is only slowly phosphorylated by chloroplasts, together with P_i can effect rapid nucleotide release, too [27].

The energy-transfer inhibitor phlorizin in contrast to its inhibitory effect on phosphorylation was reported to exhibit only low inhibition of nucleotide release [34]. These results were gained from kinetic measurements with a low time resolution. At milliseconds time resolution, however, a specific inhibition of the rapid initial release phase is apparent. Simultaneous measurements of initial release and initial phosphorylation indicates the same dependence on phlorizin concentration (Table VI). Phlorizin was found to act on the ATPase as a competitor with P_i [35]. Indeed our results could be well explained by screening of the phosphate binding site with phlorizin. Unfortunately, however, phlorizin in our hands proved to be a non-competitive inhibitor and the phlorizin effect on nucleotide release was not abolished by increasing the phosphate concentration (not shown).

Discussion

Acceleration of initial light-induced release of tightly bound nucleotides by medium ADP and phosphate requires to assume a negative cooperative effect by the interaction of these substrates with other non-occupied centers. As both substrates are necessary for maximal stimulation and ATP is formed simultaneously, it is likely that the involved free sites are catalytic sites. However, stimulation of nucleotide release requires just binding of the substrates rather than catalysis (Tables IV and V). The occurence of two distinct substrate constants for each, ADP and P_i suggests participation of two catalytic centers.

Release of a tightly bound nucleotide requires transformation of the respective 'tight site' into a 'loose site' by $\Delta\tilde{\mu}_{H^+}$-dependent conformational change [20–23]. One may imagine that the closed or buried site is opened so that the screened nucleotide can undergo dissociation. Catalytic sites are likewise unavailable in enzymes containing a tightly bound nucleotide and get accessible to medium substrates by thylakoid energization [17,36]. Probably these alterations of binding sites are the essential results of enzyme activation.

Photolabeling experiments have shown that the site where tightly bound ADP is located originates from a catalytic site [19]. This can also be concluded from the fact that some tightly bound ATP is found when loading of the site by ADP (in the presence of endogenous or added phosphate) is conducted in the light (Table I) and that tightly bound ADP is detected when loading is performed with ATP [45]. At very high phosphate concentration and low pH tightly bound ADP can serve as phosphate acceptor in ATP formation without requirement of energy [43,44]; when membranes are energized in the presence of physiological phosphate concentrations and ADP, however, the bound ADP is expelled rather than phosphorylated (Table III).

The results of this paper are in agreement with the view that activation by light of high intensity primarily induces a condition of the ATPase with three principally identical open sites which are potential catalytic centers. Presumably one site is located on one α–β subunit pair. Nevertheless, the enzyme exhibits asymmetry as one of the sites is occupied with ADP (or ATP). On the basis of measurements of enzyme activity as function of ΔpH it was concluded that the activation process takes place stepwise in a defined sequence and that one H^+ reacting from the lumen side of the thylakoid is necessary to activate one subunit entity [37]. The ratio of enzyme forms with zero, one, two or three activated centers depends on the magnitude of $\Delta\tilde{\mu}_{H^+}$. For the evolution of catalytic activity opening of all three sites is necessary when one of the sites is covered with a nucleotide molecule [37]. In the absence of medium substrates, the nucleotide may slowly dissociate and thus form a symmetric enzyme; in the presence of ADP and P_i, however, binding of the substrates to the non-occupied sites exerts the observed pressure on nucleotide release.

Upon energization with short light pulses we can expect that only a certain portion (which depends on light intensity) of enzymes reaches to the catalytically active state rapidly. For this initial activation, the main driving force is the membrane potential (Fig. 5a). When ADP and P_i are available, the formation of ATP begins. It is comprehensible that further activation is hindered under those conditions as the electrochemical gradient is partially consumed by phosphorylation. Since the energy barrier for activation is higher than for phosphorylation [38–40], the formation of ATP would have precedence over further activation. Deceleration of activation is reflected by slower release of tightly bound nucleotides after the first 50 ms of illumination.

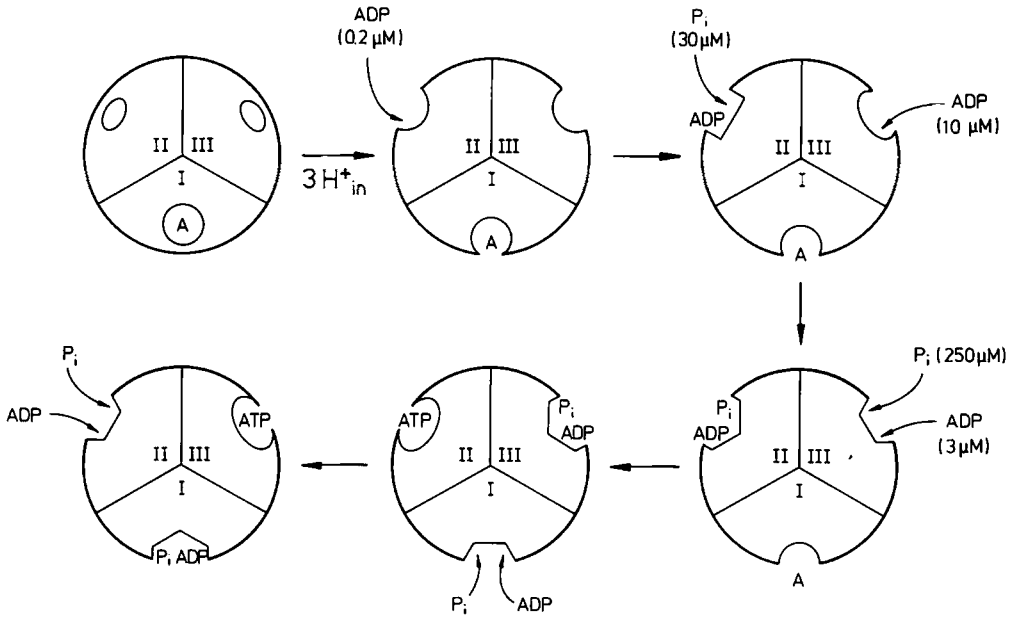

Fig. 6. Schematic presentation of the reaction steps involved in the transition of an inactive to an active ATPase. A signifies an ADP or ATP molecule; the numbers in parentheses give apparent dissociation constants for the indicated substrates at the respective sites.

Stimulation of $\Delta\tilde{\mu}_{H^+}$-induced release of the tightly bound nucleotide by medium substrates is an indicator reaction which allows conclusions about the properties of the two involved free sites. In a model shown in Fig. 6, the three centers are designated I, II and III, where I is the nucleotide-containing site. The extents of mutual influences on affinities of the sites for ADP and P_i may be estimated from the plots shown in Figs. 2 and 3. These results lead to the following conclusions.

The effect of low medium ADP concentrations on initial nucleotide release from site I may be interpreted to indicate high-affinity binding of ADP at site II. The affinity for ADP is independent of the absence or presence of phosphate. Binding of ADP at site II exerts some negative cooperation on site I, but has a strong negative cooperative effect on the ADP binding site of center III. When center II is occupied by ADP alone, the affinity for ADP at center III is lowered by a factor of 50. However, when P_i is also bound (with high affinity) at site II, the decrease of ADP affinity in center III is partially abolished. On the other hand, binding of phosphate at site II lowers the affinity for phosphate in center III. The negative cooperative effect on site I is strongest when centers II and III are both occupied with ADP and phosphate.

The indirectly estimated dissociation constant for phosphate at center III is close to the Michaelis constant for phosphate in photophosphorylation [41]. In contrast, the dissociation constants for ADP neither on site II nor on site III are in agreement with the Michaelis constant in steady-state phosphorylation. Recently, the true K_m (ADP) measured at excess phosphate and constant $\Delta\tilde{\mu}_{H^+}$ was determined as 60 μM [42]. The discrepancy between K_m and K_d, however, does not disprove that binding of medium ADP in the pre-steady-state phase occurs at catalytic sites. It has to be taken into account that K_m is a complex constant which contains several other velocity constants in addition to K_d [42].

Hence the processes observed at the transition from dark to light seem to include reactions at all three catalytic centers in the pre-steady-state phase of photophosphorylation. On the other hand, Larson et al. [47] demonstrated sulfite-stimulated ATP hydrolysis to proceed without rapid release of the tightly bound nucleotide, suggesting different pre-steady-state reactions under these particular conditions. However, neither these nor our results can answer the question whether three sites or less are actually involved in the steady-state catalytic cycle. Recent measurements of fluorescence energy transfer between fluorochromes specifically introduced into isolated CF_1, led to the conclusion that 'site 1' (which corresponds to the 'tight site') and 'site 3' (which is a catalytic site) can switch their properties as a result of catalysis [46]. These findings could be interpreted to indicate randomization of asymmetry but do not exclude a three-site catalytic mechanism as proposed by Boyer and his colleagues [8]. Anyhow they show that the 'tight site' after activation can be reintegrated in the catalytic process.

On the basis of the three-site mechanism, the transition from pre-steady-state to steady state could be interpreted as depicted in the lower part of Fig. 6. Binding of ADP and P_i to center III induces final ejection of the nucleotide from site I while ATP is synthesized in center II. In this condition site I is a

catalytic center ready for substrate binding. After binding of ADP and P_i to center I and simultaneous conversion of centers II and III to the respective next stages the enzyme has attained its steady-state working condition. It must be emphasized, however, that this part of the model of Fig. 6 is speculation rather than interpretation of the preceding results.

Acknowledgements

This work was supported by Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 189) and by Fonds der Chemischen Industrie.

References

1 Strotmann, H. and Bickel-Sandkötter, S. (1984) Annu. Rev. Plant Physiol. 35, 97–120.
2 Galmiche, J.M., Girault, G. and Lemaire, C. (1985) Phytochem. Phytobiol. 41, 707–713.
3 McCarty, R.E. and Hammes, G.G. (1987) Trends Biochem. Sci. 12, 234–237.
4 Czarnecki, J.J., Abbott, M.S. and Selman, B.R. (1982) Proc. Natl. Acad. Sci. USA 79, 7744–7748.
5 Bar-Zvi, D., Tiefert, M.A. and Shavit, N. (1983) FEBS Lett. 160, 233–238.
6 Abbott, M.S., Shavit, N., Selman-Reimer, S. and Selman, B.R. (1986) FEBS Lett. 209, 157–161.
7 Xue, Z., Zhou, J.M., Melese, T., Cross, R.L. and Boyer, P.D. (1987) Biochemistry 26, 3749–3753.
8 Boyer, P.D. and Kohlbrenner, W.E. (1981) in Energy Coupling in Photosynthesis (Selman, B.R. and Selman-Reimer, S., eds.), pp. 231–240, Elsevier/North-Holland, Amsterdam and New York.
9 Mitchell, P. (1985) FEBS Lett. 182, 1–7.
10 Hackney, D.D., Rosen, G. and Boyer, P.D. (1979) Proc. Natl. Acad. Sci. USA 79, 3646–3650.
11 Junge, W. (1970) Eur. J. Biochem. 14, 582–592.
12 Gräber, P., Schlodder, E. and Witt, H.T. (1977) Biochim. Biophys. Acta 461, 426–440.
13 Schlodder, E. and Witt, H.T. (1981) Biochim. Biophys. Acta 635, 571–584.
14 Schumann, J. and Strotmann, H. (1981) in Photosynthesis II. Electron Transport and Photophosphorylation (Akoyunoglou, G., ed.), pp. 881–892, Balaban International Science Service, Philadelphia, PA.
15 Dunham, K. and Selman, B.R. (1981) J. Biol. Chem. 256, 212–218.
16 Aflalo, C. and Shavit, N. (1982) Eur. J. Biochem. 126, 61–68.
17 Bickel-Sandkötter, S. (1983) Biochim. Biophys. Acta 723, 71–77.
18 Smith, L.T., Rosen, G. and Boyer, P.D. (1983) J. Biol. Chem. 258, 10887–10894.
19 Zhou, J.M., Xue, Z., Du, Z., Melese, T. and Boyer, P.D. (1988) Biochemistry 27, 5129–5135.
20 Strotmann, H. and Bickel-Sandkötter, S. (1977) Biochim. Biophys. Acta 460, 126–135.
21 Schumann, J. (1984) Biochim. Biophys. Acta 766, 334–342.
22 Strotmann, H., Bickel, S. and Huchzermeyer, B. (1976) FEBS Lett. 61, 194–198.
23 Strotmann, H., Bickel-Sandkötter, S. and Shoshan, V. (1979) FEBS Lett. 101, 316–320.
24 Shigalowa, T., Lehmann, U., Krevet, M. and Strotmann, H. (1985) Biochim. Biophys. Acta 809, 57–65.
25 Sugino, Y. and Miyoshi, Y. (1964) J. Biol. Chem. 239, 2360–2364.
26 Strotmann, H. (1972) in Proceedings in the IInd International Congress on Photosynthesis Research (Forti, G., Avron, M. and Melandri, A., eds.), Vol. 2, pp. 1319–1328, Dr. W. Junk N.V. Publishers, Dordrecht.
27 Strotmann, H. (1984) in Advances in Photosynthesis Research (Sybesma, C., ed.), Vol. II, pp. 477–484, Martinus Nijhoff/Dr. W. Junk Publishers, Dordrecht.
28 Rosen, G., Gresser, M., Vinkler, C. and Boyer, P.D. (1979) J. Biol. Chem. 254, 10654–10661.
29 Ort, D.R. and Dilley, R.A. (1976) Biochim. Biophys. Acta 449, 95–107.
30 Rosing, J., Smith, D.J., Kayalar, C. and Boyer, P.D. (1976) Biochem. Biophys. Res. Commun. 72, 1–8.
31 Bickel-Sandkötter, S. (1985) Biochim. Biophys. Acta 809, 117–124.
32 Avron, M. and Jagendorf, A.T. (1959) J. Biol. Chem. 234, 967–972.
33 Hall, D.O. and Telfer, A. (1969) in Progress in Photosynthesis Research (Metzner, H., ed.), Vol. III, pp. 1281–1287, H. Laupp Jr., Tübingen.
34 Bickel-Sandkötter, S. and Strotmann, H. (1976) FEBS Lett. 65, 102–106.
35 Izawa, S., Connolly, T.N., Winget, G.D. and Good, N.E. (1966) Biochem. Biophys. Res. Commun. 22, 223–226.
36 Bickel-Sandkötter, S. and Strotmann, H. (1981) FEBS Lett. 125, 188–192.
37 Lohse, D., Thelen, R. and Strotmann, H. (1989) Biochim. Biophys. Acta 976, 85–93.
38 Mills, J.D. and Mitchell, P. (1984) Biochim. Biophys. Acta 764, 93–104.
39 Junesch, U. and Gräber, P. (1985) Biochim. Biophys. Acta 809, 429–434.
40 Biaudet, P. and Haraux, F. (1987) Biochim. Biophys. Acta 893, 544–556.
41 Quick, W.P. and Mills, J.D. (1988) Biochim. Biophys. Acta 932, 232–239.
42 Heinen, G. and Strotmann, H. (1989) Z. Naturforsch., in press.
43 Feldman, R.I. and Sigman, D.S. (1982) J. Biol. Chem. 257, 1676–1683.
44 Feldman, R.I. and Sigman, D.S. (1983) J. Biol. Chem. 258, 12178–12183.
45 Magnusson, R.P. and McCarty, R.E. (1976) J. Biol. Chem. 251, 7417–7422.
46 Shapiro, A.B. and McCarty, R.E. (1988) J. Biol. Chem. 263, 14160–14165.
47 Larson, E.M., Umbach, A. and Jagendorf, A.T. (1989) Biochim. Biophys. Acta 973, 78–85.